

# Concordance between morphology and mitochondrial phylogenetic structure in Australian dobsonflies (Megaloptera: Corydalidae)

A.M. BAKER & G. THEISCHINGER<sup>1</sup>

**Abstract:** A first-order comparison of morphology and mitochondrial phylogenetic structuring is conducted in representative dobsonfly (Megaloptera: Corydalidae) taxa, occurring in the Sydney Water Supply Catchment, south-eastern Australia. The current morphological classifications of the taxa examined are concordant with relationships inferred from the phylogenetic data. However, estimated divergence times between pairs of taxa based on morphology and biogeographic events, pre-date times inferred from genetic data. Possible reasons for this discrepancy are discussed, and the genetic results are compared with those obtained previously for other macroinvertebrate taxa occurring in the study area.

**Key words:** phylogeny, mitochondrial DNA, morphology, Megaloptera, Corydalidae, macroinvertebrate.

## Introduction

Freshwater catchments of south-eastern Australia possess generally rich and diverse macroinvertebrate faunas, although phylogenetic structuring within these assemblages is, to a large extent, poorly known.

The Sydney Water Supply Catchment (SWSC), in south-eastern New South Wales, encompasses the Hawkesbury-Nepean, Georges and Shoalhaven River Catchments and is governed by the Sydney Catchment Authority (SCA). While the primary function of the SCA is to supply water of sufficient quantity and quality to customers, it also aims to achieve the successful management of genetic and species diversity, as well as maintenance of prime habitat. In a previous study, BAKER et al. (in press 1) used genetic markers to describe the pattern and extent of phylogenetic structuring within representative macroinvertebrate genera in this region. Results indicated a large amount of cryptic diversity within a range of crustaceans and insects, given the relatively small size of the study area. Distributions of most taxa examined were associated with high altitude streams, above dam impoundments. These baseline data will be used in coming years to predict the likely consequences for bio-

diversity in the SWSC, if certain developments go ahead, and provide information on how to moderate the impacts of such development.

In the present study, we add to the previous work conducted on freshwater macroinvertebrates in the SWSC, by presenting preliminary analyses comparing morphology and mitochondrial genetic structure of corydalid megalopterans. To our knowledge, this study represents the first research investigating phylogenetic relationships among Australian megalopterans.

## Methods

### Taxa

All Australian corydalids belong in the subfamily Chauliodinae. They are rather large insects, with both adults and larvae possessing strongly developed mandibles. The adults have long antennae, long legs, two pairs of functional membranous wings and a very soft abdomen. The head of the larvae is flat and prognathous, the thorax is largely sclerotised, the legs are short, and there are lateral filaments (gills) on segments 1-8, with a pair of large prolegs on segment 10 of the abdomen.

<sup>1</sup> Unserem Kollegen und langjährigen Freund (seit dem 1949 gleichzeitig von HA, in mehrfacher Damenbegleitung, und GT, solo, durchgeführten, historischen Insektennadel-Kauf bei Quirin Haslinger in Linz) Horst Aspöck mit Respekt und in Anerkennung seiner wissenschaftlichen Leistungen gewidmet. Andrew Baker und Günther. Quirin Haslinger: Lehrmittel-Handlung mit einiger Tradition; man konnte dort von Sammlungsexemplaren und Insekten-Biologien bis zu Insektennadeln und wissenschaftlicher Literatur alles Erdenkliche kaufen und bestellen, selbst Adressen einem unbekannten Kollegen bekommen. Eine schöne Einrichtung, deren Auslagen Horst Aspöck, ich (GT) und wohl auch andere „abnormale“ Kinder mit viel Liebe und Interesse studiert haben.

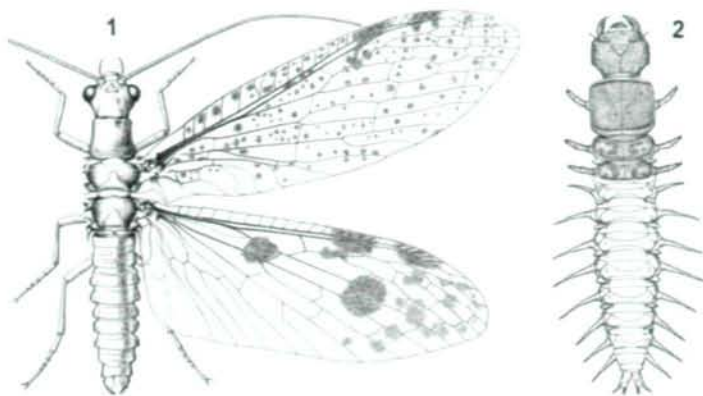


Fig. 1: *Archichauliodes guttiferus* (WALKER), adult male.

Fig. 2: *Protochauliodes biconicus* KIMMINS, final instar larva, dorsal.

Corydalidae are generally associated with clear, cold water, usually in riffle situations, but at least some are known from swamps (THEISCHINGER 1991). Adults (Fig. 1) emerge in the warmer seasons, are short-lived, take little food and during the day are usually found resting on stems of plants bordering larval habitats. There is a strong crepuscular or nocturnal tendency of the adults, as well as an attraction to artificial lights. Copulation takes place on vegetation near the water, and egg-masses are laid on rocks or vegetation overhanging or projecting from water. After hatching, the larvae (Fig. 2) drop into the water where they live as predators under rocks and debris for 1-5 years. Pupation takes place on land in a simple chamber in soil or litter. The pupal stage lasts only a few weeks.

## Study Area

The Sydney Water Supply Catchment is a network delivery system drawing water from three major catchments in south-east New South Wales, Australia: The Hawkesbury/Nepean, Georges and Shoalhaven. However, the SWSC also encompasses several eastward-flowing river catchments (Fig. 3).

## Sampling and Identification

Representative larval samples of dobsonfly taxa from selected river catchments (Fig. 3), were obtained by kick-sampling riffle habitat. These samples were immersed under liquid nitrogen, returned to the laboratory and held in a  $-80^{\circ}\text{C}$  freezer, until required for analysis.

Group taxa were identified based on external morphology, using the available literature (THEISCHINGER 1999, 2000), with the aid of a high-powered stereo microscope.

## Molecular Procedures

Total genomic DNA was isolated using a modification of the CTAB/phenol-chloroform DNA extraction protocol (DOYLE & DOYLE 1987). PCR was undertaken using mitochondrial coding region cytochrome c oxidase subunit I (COI) primers LCO1490 and HCO2198 (FOLMER et al. 1994).

50  $\mu\text{L}$  total volume PCR reactions contained: 35.8  $\mu\text{L}$  ddH<sub>2</sub>O, 2  $\mu\text{L}$  each of 10  $\mu\text{M}$  primers, 1  $\mu\text{L}$  10 mM dNTPs, 2  $\mu\text{L}$  50 mM MgCl<sub>2</sub>, 5  $\mu\text{L}$  10X Reaction Buffer, 0.2  $\mu\text{L}$  DNA Taq polymerase and 2  $\mu\text{L}$  template DNA. PCR was performed on the Geneamp PCR System 9700 (PE Applied Biosystems) with an initial denaturation step of  $94^{\circ}\text{C}$  for 3 minutes; 30 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $50^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 30 seconds; and a final extension step of  $72^{\circ}\text{C}$  for 5 minutes.

PCR products were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced, using both heavy and light strand primers, on an Applied Biosystems 377 automated sequencer, with complete overlap of sequences, to ensure DNA-strand homology.

Sequences were aligned using BioEdit Version 5.0.9 (HALL 1999). Estimates of p-distance and net (corrected) pairwise sequence diversity  $\pm$  SE (10,000 bootstraps) were calculated using MEGA Version 2.1 (KUMAR et al. 2001). Genetic distances for calculation of net sequence diversity between taxa, were calculated

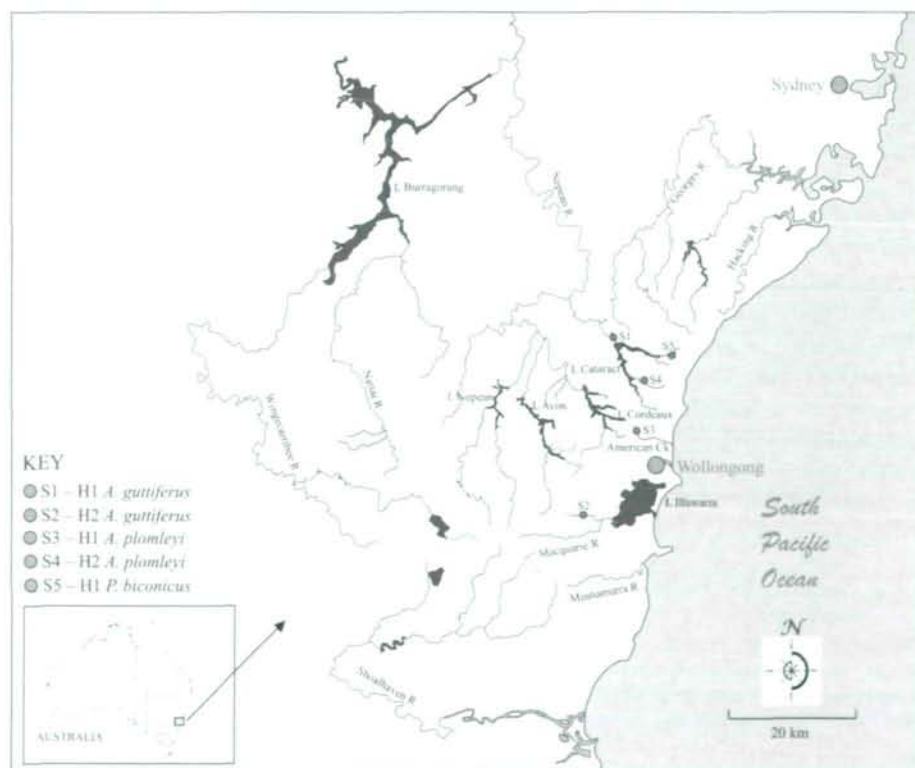


Fig. 3: Sample sites (Key indicates site of origin for each haplotype).



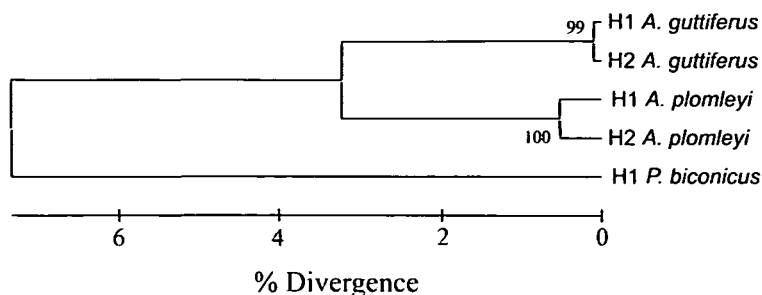


Fig. 10: Midpoint-rooted Neighbor-Joining phylogeny. Scale indicates divergence and bootstrap percentages are printed at nodes.

We describe five unique COI 486 base pair haplotypes (Table 1). There were no insertions or deletions detected in the sequences. Of the 486 base pairs of unambiguous sequence, 78 sites (16%) exhibited variation and 28 sites (6%) were phylogenetically informative. Of the 28 informative sites, 1 (4%) was at the first position of the codon and 27 (96%) were at the third position. The transition (Ti) to transversion (Tv) substitution ratio was 0.8:1.

Uncorrected pairwise p-distance sequence divergences range from 0.3 – 1.1% within taxa and 5.8 – 14.2% between taxa (Table 2). Corrected net sequence divergences between pairs of taxa were as follows: *A. guttiferus* – *A. plomleyi* (10.14%  $\pm$  2.86); *A. guttiferus* – *P. biconicus* (43.87%  $\pm$  13.11); *A. plomleyi* – *P. biconicus* (62.19%  $\pm$  16.10).

In the present study, phylogenies were constructed under the F81 + G model of sequence evolution, with gamma shape parameter 0.0942. The Neighbor-Joining Phylogeny (Fig. 10) recovered three well-supported, divergent lineages of corydalid taxa. Maximum Likelihood and Maximum Parsimony phylogenies (not shown) recovered the same three lineages, with bootstrap support values of at least 95%. Levels of mitochondrial divergence and the positioning of phylogenetic clades are concordant with expectations, based on current morphological taxonomic designations between *Archichauliodes* species and between *Archichauliodes*/*Protochauliodes*.

The COI gene fragment did not exhibit any significant among-site rate heterogeneity (LRT (delta) = 4.26, df=3, P = 0.23). GAUNT & MILES (2002) calibrated the insect COI molecular clock, using the Blattaria/Orthoptera divergence, at 0.022% nucleotide substitutions per million years. Assuming this rate is representative of COI divergence rate in dobsonflies, and factoring the rate on the es-

timated net divergences, it can be predicted that: (1) the *Archichauliodes* species shared a common ancestor approximately 5 million years ago, at the Miocene/Pliocene boundary, and (2) *Archichauliodes guttiferus/plomleyi* and *Protochauliodes biconicus* shared a common ancestor approximately 24 million years ago, at the Oligocene/ Miocene boundary. Estimated levels of net divergence between *A. guttiferus* and *A. plomleyi* are the same order of magnitude as recorded levels of mitochondrial (COI) divergence among cryptic species of the freshwater shrimp *Paratya* (up to 8%), from the same geographic area (BAKER et al. in press 2). *Paratya* and *Archichauliodes* are co-distributed throughout much of the SWSC, and the proposed Pliocene divergence within these taxa suggests that they are likely to have been affected by the same processes.

Early Tertiary climate in Australia was warm and wet, but by the end of the Miocene Epoch the continent had become cooler and drier (CHRISTOPHEL & GREENWOOD, 1989; POLE et al. 1993). By the Pliocene, the climate throughout much of inland Australia had become semi-arid, with continental aridity increasing during the Quaternary, punctuated by pluvial intervals (ALLEY 1998). Based on the molecular data, we may therefore propose an evolutionary scenario that ancestral populations of *A. guttiferus/plomleyi* and *P. biconicus* become fragmented associated with ongoing aridity during the Miocene. *Archichauliodes* populations may have subsequently become fragmented during Pliocene aridity, evolved in isolation for a time, and recontacted during a Quaternary pluvial event.

However, notwithstanding the concordance between currently accepted classifications and molecular results in the relative systematic position of the three studied taxa within Australia, the timing of divergence based on present-day geographic distributions of these genera, is inconsistent with the genetic data. According to the morphological classification and distribution as summarized by NEW & THEISCHINGER (1993), both *Archichauliodes* and *Protochauliodes* occur in Australia and Chile, with *Archichauliodes* also in New Zealand, and *Protochauliodes* also in western North America. This suggests that a lineage including ancestral *Archichauliodes* was differentiated from a lineage including ancestral *Protochauliodes*, well before the break up of Gondwana, presumably still in the Mesozoic. This antiquity of the two genera based on their vicariant Gondwanic distribution, also suggests that the diversification of *Archichauliodes* into well-defined Australian species groups, occurred much earlier than indicated by the molecular results.

Table 2. Lower triangular matrix of uncorrected percent (p-distance) sequence divergence between pairs of haplotypes.

	H1 <i>A. guttiferus</i>	H2 <i>A. guttiferus</i>	H1 <i>A. plomleyi</i>	H2 <i>A. plomleyi</i>	H1 <i>P. biconicus</i>
H1 <i>A. guttiferus</i>	0				
H2 <i>A. guttiferus</i>	0.3	0			
H1 <i>A. plomleyi</i>	6.4	6.6	0		
H2 <i>A. plomleyi</i>	5.8	6.0	1.1	0	
H1 <i>P. biconicus</i>	12.4	12.6	14.2	14.2	0

The use of molecular clocks in evolutionary biology remains controversial, for two major reasons. First, rates across lineages, genes and genomic regions may be heterogeneous across taxa. Second, for taxa which diverged a long time ago, it is difficult to determine the actual number of nucleotide substitutions that have occurred in each lineage since common ancestry. This is because saturation (multiple substitutions at a single site) may mask the actual number of substitutions (ARBOGAST et al. 2002). Indeed, even under idealised conditions (i.e. with a clock specifically calibrated for the species under study using the fossil record), 95% confidence limits for divergence times estimated by a molecular clock, may still be quite large (HILLIS et al. 1996).

Two major factors may therefore have contributed to underestimation of divergence times, based on the present mitochondrial data:

(1) the COI gene generally exhibits a relatively fast rate of evolution which may pronounce the effects of saturation, and

(2) estimates were based on relatively few sequences from few taxa, which may reduce estimates of molecular diversity.

Potential limitations of molecular clocks notwithstanding, a better understanding of evolutionary processes affecting these genera could be attained by comparing representative sequences of all taxa within these two genera, throughout their world-wide geographic range. This would indicate how closely allied the Australian corydalid taxa are with corydalids from South America, North America and New Zealand. In addition, sequencing of more slowly evolving nuclear and/or mitochondrial genes in these taxa could reduce the effects of saturation and potentially allow the accuracy of genome-specific calibration rates to be tested.

In a previous study conducted in the SWSC, BAKER et al. (in press 1) reported high levels of genetic diversity and cryptic species within atyid shrimp (*Paratya*) and leptophlebiid mayflies (*Atalophlebia*). Given the position of the corydalids as high-order stream predators, the relatively lower levels of diversity recovered in the present study are perhaps not surprising.

However, it is not unusual for molecular, phylogenetic-based studies to inflate the number of recognised taxa in any given area. For example, over 100 new, old-world tree frog species from Sri Lanka were recognised after extensive analyses of mitochondrial DNA, morphology, ecology and bioacoustics, where previously only 18 species were known (MEEGASKUMBURA et al. 2002). In this regard, and notwithstanding the observed concordance between morphology and mtDNA, it is important to recognise that the mitochondrial gene tree may be incongruent with the organismal species trees for the iden-

tified dobsonfly lineages. Thus, the deep divergence and sympatry of mitochondrial lineages detected in the present study is certainly consistent with a scenario of historical genetic isolation, evolution along separate trajectories and subsequent recontact, but does not necessarily rule out post-separation (secondary) gene flow between them. Formal comparative analysis of nuclear (allozyme) markers would reveal whether diagnostic loci can be used as evidence for an absence of contemporary interbreeding, between sympatric lineages of corydalid taxa.

The concordance between our molecular and morphological data as well as discordance between molecular-based divergence times and biogeography, provide a fascinating starting point on the path to ultimately understanding the processes which have affected the evolution of this little known family of macroinvertebrates, in south-east Australia, or on Gondwana. It will be interesting to conduct a more extensive comparative analysis of evolutionary relationships including other groups, particularly *Protochauliodes* from Chile and *Archichauliodes* from Chile and New Zealand, in the future. It would also be valuable to conduct a cladistic analysis of morphological data for the Chauliodinae, or better still, for the Corydalidae of the world.

## Acknowledgements

We are grateful to Dr D. Bickel (Sydney) and Dr T.R. New (Melbourne) for reading the MS and giving helpful comments. Mrs S. Monteith (Brisbane) (artist) and Dr E.F. Riek (Canberra) (ex-curator) are thanked for giving us the opportunity to use illustrations done at CSIRO (Fig. 1, 2 and 4-9). We thank Arlene Wheatley (Brisbane) for lab work. AMB was funded by the Cooperative Research Centre for Freshwater Ecology (Griffith University). GT was supported by EPA (Environment Protection Authority) New South Wales, Water Science (Management Dr K. Koop and Dr P. Scanes).

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